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(54) Title: PRODUCTION OF HUMAN BASIC FGF MUTEIN

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MetProAlaLeuProGluAspGlyGlySerGlyAlaPheProProGlyHisPheLysAsp    20
ATGCCAGCATTGCCCGAGGATGGCGGCAGCGGCGCCTTCCCGCCCGGCCACTTCAAGGAC    60

ProLysArgLeuTyrCysLysAsnGlyGlyPhePheLeuArgIleHisProAspGlyArg    40
CCCAAGCGGCTGTACTGCAAAAACGGGGGCTTCTTCCTGCGCATCCACCCCGACGGCCGA   120

ValAspGlyValArgGluLysSerAspProHisIleLysLeuGlnLeuGlnAlaGluGlu    60
GTTGACGGGGTCCGGGAGAAGAGCGACCCTCACATCAAGCTACAACTTCAAGCAGAAGAG   180

ArgGlyValValSerIleLysGlyValSerAlaAsnArgTyrLeuAlaMetLysGluAsp    80
AGAGGAGTTGTGTCTATCAAAGGAGTGAGCGCTAATCGTTACCTGGCTATGAAGGAAGAT   240

GlyArgLeuLeuAlaSerLysSerValThrAspGluCysPhePhePheGluArgLeuGlu   100
GGAAGATTACTAGCTTCTAAGTCTGTTACGGATGAGTGTTTCTTTTTTGAACGATTGGAA   300

SerAsnAsnTyrAsnThrTyrArgSerArgLysTyrThrSerTrpTyrValAlaLeuLys   120
TCTAATAACTACAATACTTACCGGTCAAGGAAATACACCAGTTGGTATGTGGCACTGAAA   360

ArgThrGlyGlnTyrLysLeuGlySerLysThrGlyProGlyGlnLysAlaIleLeuPhe   140
CGAACTGGGCAGTATAAACTTGGATCCAAAACAGGACCTGGGCAGAAAGCTATACTTTTT   420

LeuProMetSerAlaLysSertrm                                       147
CTTCCAATGTCTGCTAAGAGCTGA                                       444
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(57) Abstract

Disclosed are (1) a vector comprising a nucleotide sequence coding for a mutein in which at least one constituent amin acid of a mature human basic fibroblast growth factor (hbFGF) is replaced by another amino acid, and a T7 promoter upstream therefrom; (2) a transformant transformed by the vector of (1); (3) the transformant of (2), in which a host is *E. coli* having a T7 RNA polymerase gene downstream from a lac promoter; (4) a method for producing the mutein in which at least one constituent amino acid of the mature hbFGF is replaced by another amino acid, which comprises cultivating the transformant of (2) in a culture medium; (5) the method of (4), in which about 3 to 500 μm of isopropylthiogalactopyranoside is added t the culture medium on a logarithmic growth phase of the transformant of (3), followed by cultivati n, and (6) the method of (5), in which a resultant mutein-containing solution is purified by chromatography using a crosslinked polysaccharide sulfate, a synthetic polymer having a sulfonic acid group as an exchange group and/or a synthetic polymer for gel filtration as a carrier, whereby the hbFGF mutein having biological activity can be efficiently produced.

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DESCRIPTION

PRODUCTION OF HUMAN BASIC FGF MUTEIN

Technical Field

The present invention relates to a technique for producing a mutein of a mature human basic fibroblast growth factor (hereinafter also briefly referred to as hbFGF) in which at least one constituent amino acid of hbFGF is replaced by another amino acid which can be used as a healing promoter for wounds.

Background Art

Basic fibroblast growth factor (bFGF) is a basic polypeptide hormone having a molecular weight of about 17,000 and is mainly secreted by a pituitary gland. bFGF was first isolated as a factor exhibiting strong growth promoting action on fibroblasts such as BALB/c3T3 cell [D. Gospodarowicz, Nature 249, 123 (1974)]. It is now known that FGF exhibits growth promoting action on almost all mesoblast-derived cells [D. Gospodarowicz et al., National Cancer Institute Monograph 48, 109 (1978)]. In particular, the angiogenic action of the bFGF, together with its cell growth promoting action, suggests a potential for the application thereof as a therapeutic medicine for traumas, and as a preventive and therapeutic medicine for thrombosis, arteriosclerosis, etc.

Genes coding for hbFGFs have been cloned by Abraham et al. [The EMBO Journal 5, 2523-2528 (1986)] and Kurokawa et al. [FEBS Letters 213, 189-194 (1987)], and expressed in

animal cells [FEBS Letters 213, 189-194 (1987)], yeast [The Journal of Biological Chemistry 263, 16471-16478 (1988)] and *Escherichia coli* [The Journal of Biological Chemistry 263, 16297-16302 (1988)]. However, their production is not always sufficient and the resulting samples are too unstable to use as medicines. As a result of various studies to solve the unstability, it has been revealed that compounds in which cysteine residues of hbFGF molecules are replaced by serine residues have high stability and exhibit the same biological activity [Biochemical and Biophysical Research Communication 151, 701-708 (1988)].

Further, there has been developed a method for producing a mutein in which at least one constituent amino acid of hbFGF is replaced by another amino acid by genetic engineering techniques (European Patent Publication No. 281,822).

When recombinant proteins are produced using *E. coli*, water-insoluble inclusion bodies are frequently formed to accumulate the proteins. When the desired substances are isolated and purified from such inclusion bodies, the inclusion bodies are usually solubilized by adding protein denaturing agents. As for hbFGF muteins, however, the proteins thus solubilized are inactive and no technique for reactivating them has been established yet.

The hbFGF muteins can be used as medicines if the muteins can be accumulated in large amounts and in an active state by using genetic engineering techniques, and further

isolated and purified in high yield. It is therefore an important object to establish a method for producing the muteins at high efficiency.

In general, when gene products are produced in large amounts by using genetic engineering techniques, the selection of host-vector systems and promoters is important and differs depending on each gene. Various studies on efficient expression systems of hbFGF muteins have revealed that an E. coli gene expression system using a T7 promoter [F. W. Studier et al., Journal of Molecular Biology 189, 113-130 (1986)] is excellent for expression of the genes of the hbFGF muteins. The combination of the T7 promoter and hbFGF mutein genes is novel. This T7 promoter is known to be a strong promoter. In some cases, however, the accumulated proteins such as human interleukin-2, prolactin, form inclusion bodies, and most of them are accumulated in an inactive state. The present inventors have found the interleukin-2 case, and Paris, N. et al. have found the prolactin case [Paris, N. et al., Biotechnology and Applied Biochemistry, 12, 436-449 (1990)].

Disclosure of Invention

The present inventors conducted intensive investigations to accumulate considerable amounts of the hbFGF mutein in an active state, and have found that addition of isopropylthiogalactopyranosyde in a low concentration is effective. In addition, the present inventors have further established a method for isolating

and purifying the hbFGF mutein efficiently, thus completing the present invention.

The present invention provides:

(1) a vector containing a nucleotide sequence coding for a mutein in which at least one constituent amino acid of a mature human basic fibroblast growth factor (hbFGF) is replaced by another amino acid, and a T7 promoter upstream therefrom,

(2) a transformant transformed by the vector described in the above item (1),

(3) the transformant described in the above item (2), in which a host is *E. coli* having a T7 RNA polymerase gene downstream from a lac promoter,

(4) a method for producing the mutein in which at least one constituent amino acid of the mature hbFGF is replaced by another amino acid, which comprises cultivating the transformant described in the above item (2) in a culture medium,

(5) the method described in the above item (4), in which about 3 to 500 μM of isopropylthiogalactopyranoside (hereinafter also briefly referred to as IPTG) is added to the culture medium on a logarithmic growth phase of the transformant described in the above item (3), followed by cultivation, and

(6) the method described in the above item (5), in which a resultant mutein-containing solution is purified by chromatography using a crosslinked polysaccharide sulfate, a

synthetic polymer having a sulfonic acid group as an exchange group and/or a synthetic polymer for gel filtration as a carrier.

According to the present invention, the substitution type muteins of mature hbFGFs can be efficiently produced. The present invention can therefore be advantageously used for industrial production of the muteins.

Brief description of the Drawings

Fig. 1 shows a DNA nucleotide sequence of rhbFGF mutein CS23 used in Example 1 and an amino acid sequence of a protein for which the nucleotide sequence codes;

Fig. 2 is a schematic representation showing the construction of plasmid pTB960 obtained in Example 1;

Fig. 3 shows a SDS-PAGE pattern of a purified sample obtained in Example 5 and a marker; and

Figs. 4 to 6 show patterns of high performance liquid chromatography of the purified sample obtained in Example 5.

Figs. 7 to 9 are schematic representations showing the construction of plasmids pHP901, pME901 and pCM901 obtained in Example 8.

Detailed Description of the Inventions

The mature hbFGF in the present invention is a peptide consisting of 146 amino acids, counting the amino acid Pro next to Met of the N-terminus as the 1st and the amino acid Ser of the C-terminus as the 146th, in Fig. 1.

Examples of the hbFGF muteins as shown in the present invention include muteins in which at least one constituent

amino acid of the mature hbFGF is replaced by another amino acid, as described in European Patent Publication No. 281,822 and Biochemical and Biophysical Research Communication 151, 701-708 (1988).

As to the number of hbFGF-constituent amino acids before substitution in the mutein, which has at least one hbFGF-constituent amino acid substituted by another amino acid, it may be any number as long as FGF characteristics, such as the characteristics of angiogenesis, cell growth stimulating activity and cell differentiating activity, are not lost.

Examples of the constituent amino acids before substitution include cysteine and amino acids other than cysteine. In particular, cysteine is preferred. The amino acids other than cysteine as the constituent amino acids before substitution include aspartic acid, arginine, glycine and valine.

When the constituent amino acid before substitution is cysteine, neutral amino acids are preferred as the substituting amino acids. Specific examples of the neutral amino acids include glycine, valine, alanine, leucine, isoleucine, tyrosine, phenylalanine, histidine, tryptophan, serine, threonine and methionine. In particular, serine and threonine are preferred.

When the constituent amino acid before substitution is an amino acid other than cysteine, there are selected as the other substituting amino acids, for example, amino acids

different in hydrophilicity, hydrophobicity or electric charge from the constituent amino acid before substitution. Specifically, when the amino acid before substitution is aspartic acid, the substituting amino acids include asparagine, threonine, valine, phenylalanine and arginine, Asparagine and arginine are particularly preferred.

When the amino acid before substitution is arginine, the substituting amino acids include glutamine, threonine, leucine, phenylalanine and aspartic acid. Glutamine is especially preferable.

When the constituent amino acid before substitution is glycine, the substituting amino acids include threonine, leucine, phenylalanine, serine, glutamic acid and arginine. Threonine is particularly preferable.

When the constituent amino acid before substitution is serine, the substituting amino acids include methionine, alanine, leucine, cysteine, glutamine, arginine and aspartic acid. In particular, methionine is preferred.

When the constituent amino acid before substitution is valine, the substituting amino acids include serine, leucine, proline, glycine, lysine and aspartic acid. Serine is especially preferred.

As the original constituent amino acids before substitution, aspartic acid, arginine, glycine, serine and valine are preferably selected.

As the substituting amino acids, asparagine, glutamine, arginine, threonin , methionine, serine and leucin are

preferably selected.

The most preferred substituted muteins include a mutein in which cysteine, the constituent amino acid, is replaced by serine.

In the above substitution, the substitution of at least two constituent amino acids may be simultaneously carried out. In particular, it is preferable to substitute two or three constituent amino acids.

Preferred examples of the hbFGF muteins in the present invention include a mutein in which at least one cysteine residue of the mature hbFGF mutein is replaced by a serine residue.

As the mutein, recombinant hbFGF mutein CS23 (hereinafter also briefly referred to as rhbFGF mutein CS23) is particularly preferred in which cysteine residues at the 69- and 87- positions of the mature hbFGF are replaced by serine residues, respectively. The position of the amino acids of the above hbFGF are numbered, by counting the amino acid Pro next to Met of the N-terminus of the amino acid sequence as shown in Fig. 1 as the 1st.

In order to produce the muteins, site-directed mutagenesis is employed. This technique is well known and described in R. F. Lather and J. P. Lecoq, Genetic Engineering, p.31-50, Academic Press (1983). Mutagenesis directed to oligonucleotide is described in M. Smith and S. Gillam, Genetic Engineering: Principles and Methods, vol.3, p.1-32, Plenum Press (1981).

The production of a structural gene which encodes the mutein is carried out, for example, by the steps of:

(a) hybridizing a single-stranded DNA comprising a single strand of the structural gene of the hbFGF with a mutagenic oligonucleotide primer (the above primer is complementary to a region including a codon for cysteine to be replaced by this single strand or including an anti-sense triplet which forms a pair with this codon in some cases, provided this does not apply to disparity with other codons for the amino acid than the above codon, or with the anti-sense triplet in some cases),

(b) elongating the primer with DNA polymerase to form a mutational heteroduplex, and

(c) replicating this mutational heteroduplex.

Then, phage DNA for transferring the mutagenized gene is isolated and introduced into a plasmid.

As the T7 promoter used in the present invention, there may be used any of 17 kinds of promoters discovered on T7 DNA [J. L. Oakley et al., Proc. Natl. Acad. Sci. U.S.A. 74, 4266-4270 (1977); M. D. Rosa, Cell 16, 815-825 (1979); N. Panayotatos et al., Nature 280, 35 (1979); J. J. Dunn et al., J. Mol. Biol. 166, 477-535 (1983)], but a ϕ10 promoter [A. H. Rosenberg et al., Gene 56, 125-135 (1987)] is preferably used.

As a transcription terminator used in the present invention, any terminator may be used as long as it functions in E. coli systems, but a Tϕ terminat r [F. W.

Studier et al., J. Mol. Biol. 189, 113-130 (1986) is preferably used.

The T7 RNA polymerase genes used in the present invention include T7 gene 1 [F. W. Studier et al., J. Mol. Biol. 189, 113-130 (1986)].

Examples of vectors from which the vectors used in the present invention are formed include pBR322, pUC8, pUC9, pMB9, pKC7, pACYC177 and pKN410.

The vectors used in the present invention are constructed by incorporating the T7 promoter and the T7 terminater into the above vectors. Such vectors include pET-1, pET-2, pET-3, pET-4 and pET-5 [A. H. Rosenberg, Gene 56, 125-135 (1987)], but pET-3C (ibid.) is preferably used.

As the host for the transformant used in the present invention, any of E. coli strains into which the T7 RNA polymerase gene (T7 gene 1) [F. W. Studier et al., J. Mol. Biol. 189, 113-130 (1986)] is incorporated, such as MM294, DH-1, C600 and BL21, may be used. The strains MM294 and BL21 are preferably used in which a λphage including T7 gene 1 is lysogenized. The T7 RNA polymerase gene can also be harbored as a plasmid having different origin from that of expression vector. In this case, as the promoter for T7 gene 1, there is used the lac promoter whose expression is induced with isopropyl-1-thio-β-D-galactopyranoside (IPTG).

The transformants used in the present invention are obtained by transforming the E. coli strains into which the above T7 gene 1 (RNA polymerase gene) have been

incorporated, with the plasmids having the gene-transcription terminators for T7 promoter expression, according to methods known in the art such as the methods described in Proc. Natl. Acad. Sci. U.S.A. 69, 2110 (1972) and Gene 17, 107 (1982). In this case, the hosts to be used may be preliminarily transformed with plasmids having the T7 lysozyme gene so that the resulting transformants have two kinds of different plasmids simultaneously.

When the transformants are cultivated, liquid media are particularly suitable as media used for culture. Carbon sources, nitrogen sources, inorganic compounds and others necessary for growth of the transformants are contained therein. The carbon sources include, for example, glucose, dextrin, soluble starch and sucrose. The nitrogen sources include inorganic or organic materials such as ammonium salts, nitrates, corn steep liquor, peptone, casein, casamino acids, meat extracts, soybean meal and potato extract solution. Examples of the inorganic compounds include calcium chloride, sodium dihydrogenphosphate, and magnesium chloride. Yeast extracts, vitamins, growth promoting factors and the like may be further added thereto.

The pH of the media is desirably about 6 to 8.

As the medium used for cultivation of the E. coli transformants, there is preferred, for example, M9 medium (Miller, Journal of Experiments in Molecular Genetics, 431-433, Cold Spring Harbor Laboratory, New York, 1972) supplemented with glucose and casamino acids. Iron ion

sources may be further added to this medium. The iron ion sources are materials which dissociate into iron ions in solution or materials utilized in iron ion form. Such materials include salts of iron, preferably ferrous or ferric inorganic salts such as ferrous chloride, ferric chloride, ferrous sulfate, ferric sulfate, ferric phosphate and ferric nitrate. The iron ion sources are added in an amount of about 10^{-6} to 10^{-4} M, preferably in an amount of about $5X10^{-6}$ to $5X10^{-5}$ M. The cultivation is usually carried out at about 15 to 43°C for about 3 to 72 hours, preferably for about 12 to 48 hours, with aeration or agitation if necessary.

For the gene expression, it is preferable to add IPTG in the course of cultivation. Thereby, T7 gene 1 (RNA polymerase gene) ligated downstream from the lac promoter is expressed, and T7 phage RNA polymerase 1 thus produced specifically recognize the T7 promoter. According to the prior art, IPTG is added in an amount of 0.1 to 20 mM, preferably in an amount of 1 to 2 mM for expression of the lac promoter [up to 1 mM of IPTG: Løbner-Olesen et al., Cell, 57, 881-889 (1989); 2 mM of IPTG: Ernst H. et al., Gene, 68, 345-355 (1988)]; 20 mM of IPTG: Luck, D. N. et al., DNA, 5, 21-28 (1986)]. However, it has been revealed that proteins accumulated in inducing expression by addition of IPTG of this amount form inclusion bodies, which sometimes results in accumulation of inactive proteins. As a result of various studies on methods for accumulating the

proteins in a dissolved state, it has been discovered that the object is first attained by adding about 3 to 500 μM, preferably about 3 to 300 μM, more preferably 6 to 200 μM, and most preferably about 6 to 80 μM of IPTG in the course of cultivation. When large-scale cultivation such as tank cultivation is carried out, it is preferred that IPTG is added in an amount of about 10 to 500 μM, preferably 10 to 200 μM, more preferably 10 to 100 μM, still more preferably 10 to 80 μM. When small-scale cultivation such as flask cultivation is carried out, it is preferred that IPTG is added in an amount of about 3 to 100 μM, more preferably 6 to 80 μM. IPTG is first added about 1 to 24 hours, preferably about 3 to 12 hours after the initiation of cultivation, and it is preferred that IPTG is added on a logarithmic growth phase. IPTG is hereafter added intermittently or continuously as required. The media containing IPTG are cultivated at a temperature of about 20 to 42°C, preferably about 20 to 30°C. In lieu of isopropylthiogalactopyranoside, there are used in some cases, for example, propylthiogalactopyranoside, methylthiogalactopyranoside, butylthiogalactopyranoside and cyclohexylthiogalactopyranoside.

The hbFGF muteins in the present invention can be isolated and purified from the cultures obtained above, for example, by the following methods.

When the hbFGF muteins in the present invention are extracted from the cultivated cells, the cells are collected

after cultivation by methods known in the art, such as centrifugation. Then the collected cells are subjected to disruption by glass beads, French press, ultrasonic treatment, lysozyme treatment and/or freeze-thawing. In particular, the disruption by glass beads is preferable.

Various studies have been made on methods for purifying the hbFGF muteins according to the present invention from the supernatants obtained above. As a result, high-purity samples have been obtained in very high yield by combining affinity chromatography using a crosslinked polysaccharide sulfate as a carrier, ion exchange chromatography whose carrier is a synthetic polymer having a sulfonic acid group as an exchange group and chromatography using a synthetic polymer for gel filtration as a carrier with one another at least once.

The crosslinked polysaccharide sulfates used in the present invention include crosslinked cellulose sulfates, crosslinked agarose sulfates and crosslinked dextran sulfates.

The above cellulose is a polysaccharide composed of glucose linked by β-1,4 bonds, and its molecular weight is preferably about 50,000 to 2,000,000. Specific examples thereof include Avicel (crystalline cellulose, Asahi Chemical Industry, Japan) and Cellulofine (Chisso Corporation, Japan).

The above agarose is a polysaccharide which is the main component of agar, and has the recurring structure of

D-galactosyl-($\beta 1 \rightarrow 4$)-3,6-anhydro-L-garactosyl-($\alpha 1 \rightarrow 3$). Its molecular weight is preferably about 10,000 to 5,000,000. Specific examples thereof include Sepharose 2B, Sepharose 4B and Sepharose 6B (Pharmacia, Sweden).

The above dextran is a D-glucose polymer mainly comprising $\alpha(1 \rightarrow 6)$ bonds formed, for example, by the action of a microorganism such as Leuconostoc mesenteroides on sucrose. Its average molecular weight is preferably about 1,000 to 40,000,000.

The crosslinked polysaccharide sulfates used in the present invention are prepared by treating crosslinked polysaccharides such as the above dextran, agarose and cellulose with known crosslinking agents such as epichlorohydrin and 2,3- dibromopropanol according to methods known in the art.

The crosslinked polysaccharides are commercially available and can be purchased from Pharmacia (Sweden) under the trade names of Sephadex G-10, Sephadex G-15, Sephadex G-25, Sephadex G-50 and Sephadex G-100 (crosslinked dextran), and under the trade names of Sepharose CL-2B, Sepharose CL-4B and Sepharose CL-6B (crosslinked agarose). Also, crosslinked cellulose can be purchased from Chisso Corporation (Japan) under the trade name of Cellulofine (crosslinked cellulose). The desired crosslinked polysaccharide sulfates can be synthesized by allowing known sulfating agents, such as chlorosulfonic acid and sulfuric anhydride esters, to react with these crosslinked polysaccharides.

Examples of the crosslinked cellulose sulfates include the product put on the market by Seikagaku Kogyo (Japan) under the trade name of Sulfated Cellulofine (crosslinked cellulose sulfate).

Examples of the crosslinked dextran sulfates include sulfated Sephadex.

Examples of the crosslinked agarose sulfates include sulfated Sepharose.

The crosslinked polysaccharide sulfates used in the present invention may be in the form of the corresponding salts. Examples of the salts include sodium, potassium, ammonium and trimethylammonium salts. In particular, the sodium salts are preferably used.

The crosslinked polysaccharide sulfates used in the present invention are insoluble in water, and therefore it is preferred to use them in their gelatinous state by hydration.

The methods for purifying the hbFGF muteins using the crosslinked polysaccharide sulfates in the present invention include affinity chromatography described below.

hbFGF mutein-containing aqueous media are solutions containing the hbFGF muteins. The aqueous media, include water and media mainly composed of water, and are preferably adjusted to the pH range of about 3 to 10 with buffer solutions such as phosphate buffer, citrate buffer and Tris-hydrochloric acid buffer, to prevent inactivation of the hbFGF muteins.

The hbFGF mutein-containing solutions are next readjusted to a pH range of about 5.0 to 9.0, and then diluted with distilled water as is required, so that they have an electric conductivity of about 15 m℧ or less. The hbFGF mutein-containing solutions thus obtained are brought into contact with crosslinked polysaccharide sulfate gel. For this purpose, both batch and column methods may be used. The column method is however more suitable due to its simple operation. In the case of the column method, the crosslinked polysaccharide sulfate gel is filled into a column, and thereafter to equilibrate the column it is thoroughly washed with a suitable buffer solution such as 50 mM citrate buffer (pH 7.0) containing 0.4 M NaCl. The amount of the gel to be used depends on the nature of the loaded hbFGF mutein-containing solution, but the range of about 1 to 50 ml per mg of hbFGF mutein is preferable.

The hbFGF mutein-containing solutions described above are then loaded on the column. The loading speed is selected in the space velocity (SV) range of about 0.1 to 5.0. After loading, the column is thoroughly washed, and the ionic strength of the buffer solution is increased by conventional methods to elute and recover the hbFGF muteins. In order to increase the ionic strength, salts such as NaCl are added or buffer solutions high in concentration are used so that the electric conductivity is increased to at least about 15 m℧, preferably at least 30 m℧. For elution, both batch and concentration gradient elution methods may be

used. When the concentration gradient elution method is used, for example, the concentration of NaCl is gradually increased from about 0 M to 2.0 M, thereby conducting elution and recovery. Thus, highly purified hbFGF muteins can be obtained in high yield.

Examples of the synthetic polymers having sulfonic acid groups as exchange groups which are used in the present invention include polymers in which sulfonic acid groups are directly or indirectly introduced into hydrophilic vinyl polymers, styrene-divinylbenzene polymers, acrylamide polymers and the like. In particular, SP (sulfopropyl)-Toyopearl (Tosoh, Japan) in which sulfonic groups are introduced into the hydrophilic vinyl polymer is preferably used from the viewpoints of recovery and operation.

When chromatographed, the hbFGF mutein-containing solutions partially purified are adjusted to about 50 mM or less in salt concentration, for example, in the case of phosphate buffer, and allowed to be adsorbed on the above resins within the pH range of about 5 to 7. For adsorption, both batch and column systems may be used, but the column system is preferably used from the viewpoint of operation. Elution from the resins is carried out by increasing the salt concentration. For elution, both batch and concentration gradient methods may be used. When the batch method is used, for example, a buffer solution prepared by adding NaCl to the above phosphate buffer to a concentration of about 500 mM to 1 M can be used. In lieu of the

phosphate buffer, citrate buffer can be used. The elution is conducted at a temperature of about 1 to 25°C, preferably about 1 to 10°C, more preferably about 4°C.

Examples of the synthetic polymers for gel filtration used in the present invention include hydrophilic vinyl polymers and acrylamide polymers. In particular, Toyopearl HW (Tosoh, Japan), the hydrophilic vinyl polymer, is preferably used from the viewpoints of gel durability and operation.

When the hbFGF mutein-containing solutions partially purified are treated, for example, on a column of Toyopearl HW-50F, buffer solutions such as phosphate buffer and citrate buffer can be used as developing solvents. It is however advantageous to use 50 mM citrate buffer (pH 7.0). The treatment is conducted at a temperature of about 1 to 25°C, preferably about 1 to 10°C, more preferably about 4°C.

A method other than the above methods may be used as one of the purifying procedures. In such methods, for example, natural products such as cellulose, agarose and dextran and inorganic materials such as glass beads can also be used as carriers for ion exchange chromatography.

As carriers for gel filtration, there can be similarly used gels mainly composed of natural products such as cellulose, agarose and dextran and gels based on inorganic materials such as glass beads.

The samples thus obtained can also be dialyzed and lyophilized to form dried powders. Further, it is suitable

to add serum albumin as a carrier to the samples to store them, because the samples can be prevented from being adsorbed on vessels.

The coexistence with trace amounts of reducing agents in the course of purification or storage is suitable to prevent the samples from being oxidized. The reducing agents include β-mercaptoethanol, dithiothreitol and glutathione.

According to the present invention, substantially pure hbFGF muteins, essentially free from pyrogens and endotoxins can be obtained. The substantially pure hbFGF muteins according to the present invention include products which contain the hbFGF muteins according to the present invention in an amount of 95% (w/w) or more as protein content, more preferably in an amount of 98% (w/w) or more.

The hbFGF muteins obtained by the above methods of the present invention have fibroblast growth promoting activity, vascular endothelial cell growth promoting activity and angiogenic activity, are high in stability and have low toxicity. They can therefore be used as healing promoters for burns, wounds, postoperative tissues and the like, or as therapeutic medicines based on their angiogenic activity for thrombosis, arteriosclerosis and the like. They can also be used as reagents for promoting cell cultivation. In particular, the mutein in which at least one of constituent cysteine residues is replaced by a serine residue is preferable because of their high stability.

When the hbFGF muteins according to the present invention are used as pharmaceutical preparations, they can be safely administered parenterally or orally to warm-blooded animals (such as humans, mice, rats, hamsters, rabbits, dogs and cats), in a powder form as such, or as pharmaceutical compositions (such as injections, tablets, capsules, solutions and ointments) with pharmacologically acceptable carriers, excipients and diluents.

The injections are prepared by conventional methods using, for example, physiological saline or aqueous solutions containing glucose or other auxiliary agents. The pharmaceutical compositions such as tablets and capsules can also be prepared in accordance with conventional methods.

When the hbFGF muteins according to the present invention are used as the above pharmaceutical preparations, they are administered, for example, to the above warm-blooded animals in an appropriate amount ranging from about 1 ng/kg body weight to 100 μg/kg body weight daily, taking into account the route of administration, symptoms, etc.

Further, when the hbFGF muteins according to the present invention are used as the reagents for accelerating cell cultivation, they are preferably added to culture media so as to be contained in an amount of about 0.01 to 10 μg per liter of medium, more preferably in an amount of about 0.1 to 10 μg per liter of medium.

When bases, amino acids and so on are indicated by th abbreviations in this specification and the drawings, the

abbreviations adopted by IUPAC-IUB Commission on Biochemical Nomenclature or commonly used in the art are employed. For example, the following abbreviations are used. When the optical isomer is capable of existing with respect to the amino acids, the L-form is represented unless otherwise specified.

DNA : Deoxyribonucleic acid
cDNA : Complementary deoxyribonucleic acid
A : Adenine
T : Thymine
G : Guanine
C : Cytosine
RNA : Ribonucleic acid
dATP : Deoxyadenosine triphosphate
dTTP : Deoxythymidine triphosphate
dGTP : Deoxyguanosine triphosphate
dCTP : Deoxycytidine triphosphate
ATP : Adenosine triphosphate
Tdr : Thymidine
EDTA : Ethylenediaminetetraacetic acid
SDS : Sodium dodecyl sulfate
Gly : Glycine
Ala : Alanine
Val : Valine
Leu : Leucine
Ile : Isoleucine
Ser : Serine

Thr : Threonine

Cys : Cysteine

Met : Methionine

Glu : Glutamic acid

Asp : Arginine

Lys : Lysine

Arg : Arginine

His : Histidine

Phe : Phenylalanine

Tyr : Tyrosine

Trp : Tryptophan

Pro : Proline

Asn : Asparagine

Gln : Glutamine

Referring to Fig. 1, with respect to the number of the hbFGF-constituent amino acids, Met of the N-terminus of the amino acid sequence is numbered as the 1st. In this specification, however, Pro next to the Met is numbered as the 1st.

Transformant E. coli MM294/pTB762 carrying plasmid pTB762 used in Example 1 described below, transformant E. coli MM294(DE3)/pTB960 produced in Example 1, transformant E. coli MM294(DE3)/pCM901 produced in Example 8 and E. coli DH1/pTB1004 which harbors plasmid pTB1004 (Example 1) have been deposited with the Institute for Fermentation, Osaka (IFO), Japan, and with the Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of

International Trade and Industry (FRI), Japan. Their accession numbers and deposit dates are shown in Table 1. The deposit of E. coli MM294/pTB762 in FRI was initially made under accession numbers denoted by FERM P numbers. Said deposit was converted to the deposit under the Budapest Treaty and the transformants have been stored at FRI under accession numbers denoted by FERM BP numbers.

Table 1

Transformant	IFO	FRI
E. coli MM294/ pTB762	IFO 14613 (May 27, 1987)	FERM P-9409 (June 11, 1987) FERM BP-1645
E. coli MM294 (DE3)/pTB960	IFO 14979 (December 12,1989)	FERM BP-2690 (December 16,1989)
E. coli MM294 (DE3)/pCM901	IFO 15104 (October 26, 1990)	FERM BP-3168 (November 17, 1990)
E. coli DH1 /pTB1004	IFO 14827 (February 2, 1989)	FERM BP-2283 (February 13, 1989)

The present invention will hereinafter be described in detail with the following Examples. It is understood of course that these are not intended to limit the scope of the invention.

Best Mode for Carrying Out the Invention

Example 1

Preparation of rhbFGF Mutein CS23 Production Recombinant

Plasmid pTB762 containing a gene coding for rhbFGF mutein CS23 in which the cysteine residues at positions 69

and 87 of four cysteine residues existing in hbFGF were replaced by serine residues [Senoo et al., Biochemical and Biophysical Research Communication 151, 701- 708 (1988), European Patent Publication No. 281,822] was treated with EcoRI and PstI to cut out a DNA fragment coding for rhbFGF mutein CS23. Then, plasmid pTB1004 (European Patent Publication No. 336,383) was completely digested with EcoRI and PstI, and thereafter this fragment was ligated to the resulting largest fragment by T4 DNA ligase to construct plasmid pTB921. Then, the plasmid pTB921 was cleaved with restriction enzyme EcoRI, and mungbean (yaenari) nuclease was reacted with the product to change the termini thereof to flush ends, followed by cleavage with restriction enzyme BglII to prepare a DNA fragment containing the gene coding for rhbFGF mutein CS23. Additionally, vector pET3C carrying a ϕ10 promoter for a T7 phage [F. W. Studier et al., Journal of Molecular Biology 189, 113-130 (1986)] was cleaved with restriction enzyme NdeI, and the product was treated with mungbean nuclease to change the termini thereof to flush ends, followed by reaction with restriction enzyme BamHI. A DNA fragment containing rhbFGF mutein CS23 was ligated to the resulting DNA fragment by T4 ligase to obtain expression plasmid pTB960 (Fig. 2).

E. coli μM294 was lysogenized with λphage DE3 in which an RNA polymerase gene of the T7 phage was inserted [F. W. Studier et al., Journal of Molecular Biology 189, 113-130 (1986)] to prepare *E. coli* strain MM294(DE3). This

E. coli strain was transformed by using the expression plasmid pTB960, thereby obtaining recombinant *E. coli* MM294(DE3)/pTB960 (IFO 14979, FERM BP-2690) carrying the gene coding for rhbFGF mutein CS23 shown in Fig. 1.

Example 2

30 ml of LB medium (10 g/l of bactotryptone, 5 g/l of bactoyeast extract and 5 g/l of NaCl) containing 50 mg/l of sodium ampicillin was inoculated with one loopful of the recombinant *E. coli* MM294(DE3)/pTB960 (IFO 14979, FERM BP-2690), and incubated at 37°C overnight with shaking. 1.5 ml portions of this culture solution were transferred to 30 ml of M-9 medium (16.8 g/l of $Na_2HPO_4 \cdot 12H_2O$, 3 g/l of KH_2PO_4, 1 g/l of NH_4Cl, 0.5 g/l of NaCl and 0.246g/l of $MgSO_4 \cdot 7H_2O$) supplemented with 15 g/l of glucose, 15 g/l of casamino acids, 1 mg/l of thiamine hydrochloride and 50 mg/l of sodium ampicillin, and the cultivation with shaking was carried out at 37°C. When the culture was grown to a Klett unit of 100 to 120 in turbidity, IPTG was added to obtain various concentrations and the cultivation was further continued for 4 hours. Cells were collected by centrifugation and stored at -20°C.

Two identical samples were prepared. To one sample was added 7 M guanidine hydrochloride, and the cells were sufficiently loosened, followed by standing for 1 hour. Then, a supernatant was obtained by centrifugation (total amount of bFGF mutein). To the other sample was added a 50 µg/ml lysozyme solution (10% sucrose, 10 mM EDTA, 100 mM

NaCl, 1 mM APMSF and 10 mM Tris-HCl, pH 7.6), and the mixture was allowed to stand at 4°C for 1 hour. Then, the cells were disrupted by ultrasonication (Kubota Insonater 200 M), followed by centrifugation to obtain a supernatant (amount of soluble hbFGF mutein). Both the extracted solutions were diluted, and then the amount of rhbFGF mutein CS23 was determined using an ELISA. The results are shown in Table 2.

Table 2

Amount of added IPTG (μM)	Amount of accumulated rhbFGF mutein CS23 (mg/l)	Formation ratio of soluble rhbFGF mutein CS23 (%)
400	35	13
100	41	12
75	62	25
50	73	28
25	81	32
6.3	96	60
3.2	15	80
1.6	< 5	-

As is shown above, in the case of small-scale cultivation such as cultivation in flasks, the effect of the present invention can be obtained in the range of small amounts of added IPTG. Namely, about 1 mM of IPTG was added to induce the lac promoter. When IPTG was added in an amount of 100 μM or more, the productivity was low and most of the desired product was accumulated in an insoluble

state. In contrast, according to the present invention in which IPTG was added within a range from 3 μM to 100 μM, especially 6 μM to 80 μM, there were obtained the entirely unexpected results of significant improvements in productivity and also in ratio of the mutein accumulated as a soluble protein.

Example 3

M-9 medium (pH 6.8) containing 15 g/l of glucose, 15 g/l of casamino acids and 5 mg/l of thiamine hydrochloride was placed in an amount of 2.5 liter in a 5 liter jar fermentor, and sterilized. Then, the medium was inoculated with 125 ml of a strain culture solution prepared in the manner described in Example 2, followed by cultivation adjusting the pH to 6.8, at 37°C, with aeration at a rate of 2.5 l/min and with stirring at 1,000 rpm. When the turbidity reached 120 Klett units during cultivation (3 hours after the initiation of cultivation), IPTG was added so as to be contained in an amount of 10 μM. 8 hours after the initiation of cultivation, glucose and casamino acids were further added thereto so as to be contained in an amount of 10 g/l, repectively. The cultivation was further continued for 15 hours as a whole. As a result, 440 mg/l of rhbFGF mutein CS23 was accumulated in a soluble state. In contrast, when 400 μM of IPTG was added under the same conditions, the production of rhbFGF mutein CS23 was only 17.5 mg/l.

Example 4

A seed culture solution prepared in the manner described in Example 2 was transferred to a 5 liter jar fermentor in which the same medium as with Example 3 was placed, and the cultivation was initiated at 30°C, at pH 6.8, with aeration at a rate of 2.5 l/min and with stirring at 1,000 rpm. When the turbidity reached about 700 Klett units 7 hours after the initiation of cultivation, 42 μM of IPTG was added. 8.5 hours after the initiation of cultivation, 20 g/l of glucose and 20 g/l of casamino acids were added at pH 6.8. The cultivation was carried out for 36 hours. In the culture solution, 860 mg/l of rhbFGF mutein CS23 was accumulated in a soluble state.

Example 5

M-9 medium (pH 6.8) containing 15 g/l of glucose, 15 g/l of casamino acids and 5 mg/l of thiamine hydrochloride was placed in an amount of 2.5 liter in a 5 liter jar fermentor, and sterilized. Then, iron ions were added thereto at concentrations shown in Table 3 after sterilization by filtration. The resulting medium was inoculated with 125 ml portions of a strain culture solution prepared in the manner described in Example 2, followed by cultivation adjusting the pH to 6.8, at 30°C, with aeration at a rate of 2.5 l/min and with stirring at 1,000 rpm. When the culture was grown to a Klett unit of about 300 in turbidity (about 5.5 to 6 hours after the initiation of cultivation), IPTG was added so as to be contained in an

amount of 42 μM, and at the same time the cultivation temperature was lowered to 25°C, followed by cultivation for 23.5 hours. 7 to 7.5 hours after the initiation of cultivation, each of glucose and casamino acids was added in a ratio of 15 g/l, and the pH was maintained at 6.8 during cultivation. The results are shown in Table 3.

Table 3

Amount of added iron ion	Productivity of rhbFGF mutein CS23
0	1.00
$2X10^{-6}$ M	1.42
$9X10^{-6}$ M	2.60
$3.6X10^{-5}$ M	2.06
$1X10^{-4}$ M	1.23

(Note: The amount of added iron ions indicates the amount of iron ions further added to the medium. The productivity indicates the weight ratio taking as 1 when the amount of added iron ions is 0.)

Example 6

1 liter of LB medium containing 50 mg/l of sodium ampicillin was inoculated with 1 ml of the master cells of recombinant E. coli MM294(DE3)/pTB960 (IFO 14979, FERM BP-2690), and the cultivation with shaking was carried out at 30°C for 8 hours. The total amount thereof was transferred to 20 liter of LB medium (containing 50 mg/l of sodium ampicillin) placed in a 50 liter culture tank, and th

cultivation was carried out at 30°C, under an inner pressure of 1.0 kg/cm^{2}G, with aeration at a rate of 10 l/min and with stirring at 300 rpm for 7 hours. Then, 18 liters of the resulting culture solution was transferred to 360 liter of a fermentation medium (M-9 medium containing 15 g/l of glucose, 15 g/l of casamino acids, 5 mg/l of vitamin B_1 and 5 mg/l of $FeSO_4 \cdot 7H_2O$), and the cultivation was initiated at 30°C, under an inner pressure of 1.0 kg/cm^{2}G, with aeration at a rate of 240 l/min and with stirring at 250 rpm. When the culture was grown to a Klett unit of about 1,000, IPTG was added in an amount corresponding to 100 µM, and the cultivation temperature was lowered to 25°C. At the same time, a 50 liter sterilized solution containing 18 kg of glucose and 5.4 kg of casamino acids was added at a rate of about 2 l/hour, and then the cultivation was continued for 36 hours. As a result, 1.2 g/l of rhbFGF mutein CS23 was accumulated in a soluble state. The cultivated product thus obtained was subjected to a Sharpless centrifuge to collect wet cells, and the cells were frozen at -80°C to store them.

Example 7

Purification of rhbFGF Mutein CS23

1 kg of the cells (E. coli MM294(DE3)/pTB960) freeze-stored at -80°C in Example 6 was suspended in 4 liter of a buffer containing 25 mM phosphate buffer (pH 6.0), 0.1 mM p-amidinophenylmethanesulfonyl fluoride hydrochloride (APMSF) and 2 mM dithiothreitol (DTT). The cells were disrupted 5 cycles under ice cooling with a 5 liter Dynomill

Model KD-5 (Willybachfen, Switzerland) using 4 liter of glass beads. The glass beads were washed with about 5 liter of the buffer, and the washings were collected together with the extract. About 10 liter of the resulting solution was centrifuged at 10,000 rpm (Model 21, Beckman, U.S.A.) for 60 minutes to give a supernatant. The supernatant thus obtained was poured into a column (25.2 cm ID X 50 cm) of Sulfated Cellulofine (Seikagaku Kogyo, Japan). After adsorption, the column was washed with 25 liter of 25 mM phosphate buffer (pH 7.0) containing 0.5 M NaCl. Then, elution was effected using 30 liter of 25 mM phosphate buffer (pH 7.0) containing 1 M NaCl. The eluate was concentrated using an ultrafilter (Pellicon Casset System, Millipore, U.S.A.) until the absorbance at 280 nm became 3 to 4. The resulting concentrated solution was dialyzed against about 60 liter of 25 mM phosphate buffer (pH 6.0) overnight. The dialysate was centrifuged with a centrifuge (Beckman, U.S.A.) at 4,200 rpm for 30 minutes to give a supernatant. The supernatant was poured into a column (17.0 cm ID X 33.0 cm) of SP-Toyopearl 650M (Tosoh, Japan). The column was washed with 7 liter of 25 mM phosphate buffer (pH 6.0) containing 200 mM NaCl, and then elution was conducted using 10 liter of 25 mM phosphate buffer (pH 6.0) containing 500 mM NaCl. The eluate was poured into a column (15.0 cm ID X 50.0 cm) of Sulfated Cellulofine (Seikagaku Kogyo) using a high performance liquid chromatography apparatus (Gilson, France). Then, elution was effected by a linear

gradient between 20 liter of 50 mM citrate buffer (pH 7.0) and 30 liter of 50 mM citrate buffer (pH 7.0) containing 2 M NaCl. The main fractions were collected, and diluted with 10 mM citrate buffer (pH 6.0) to a conductivity of 20 mmho or less. The diluted solution was poured into a column (14.0 cm·ID X 39.0 cm) of SP-Toyopearl 650 M (Tosoh, Japan). The column was washed with 10 liter of 25 mM citrate buffer (pH 6.0), and then elution was conducted by using 10 liter of 25 mM citrate buffer (pH 6.0) containing 0.5 M NaCl. The eluate was poured into a column (14.0 cm ID X 90.0 cm) of Toyopearl HW-50F (Tosoh, Japan), and developed by using 50 mM citrate buffer (pH 7.0). The main fractions were collected, and sterilized by passing through a Millipak 20 filter (Millipore, U.S.A.) to provide a purified stock solution of rhbFGF mutein CS23. The yield was 17.0 g (the percent yield was 70%). The results of N-terminal amino acid sequence analysis, C- terminal amino acid analysis and amino acid composition analysis are shown in Tables 4 to 6, respectively.

Table 4 N-terminal amino acid sequence analysis

Cycle No.	Amino acid residue (pmol) rhbFGF mutein CS23 (750 pmol)	Amino acid deduced from nucleotide sequence
1	Pro (530)	Pro
2	Ala (553)	Ala
3	Leu (488)	Leu
4	Pro (433)	Pro
5	Glu (228)	Glu
6	Asp (269)	Asp
7	Gly (370)	Gly
8	Gly (470)	Gly
9	Ser (8)	Ser
10	Gly (328)	Gly
11	Ala (283)	Ala
12	Phe (341)	Phe
13	Pro (181)	Pro
14	Pro (338)	Pro
15	Gly (279)	Gly
16	His (69)	His
17	Phe (146)	Phe
18	Lys (264)	Lys
19	Asp (104)	Asp
20	Pro (125)	Pro

* Analyzed with a Model 477A protein sequencer (Applied Biosystems)

Table 5 C-terminal amino acid analysis

C-terminal amino acid	Yield (%)
Ser	41.0

* Hydrazinolysis method

** Analyzed with a Model 6330 amino acid analyzer (Beckman)

Table 6 Amino acid composition analysis

Amino acid	rhbFGF mutein CS23	Theoretical value
Asp/Asn	11.9	12
Thr	4.8	5
Ser	10.7	12
Glu/Gln	12.3	12
Pro	9.0	9
Gly	14.9	15
Ala	9.0	9
Cys	-	2
Val	6.4	7
Met	2.1	2
Ile	3.8	4
Leu	13.3	13
Tyr	5.9	7
Phe	8.0	8
His	3.1	3
Lys	14.2	14
Arg	10.6	11
Trp[1)]	1.1	1

\- Not assayed

* Hydrolysis method with hydrochloric acid

** Analyzed with a Model 6330 amino acid analyzer (Beckman)

1) Edelhoch method

Further, Fig. 3 shows the results of SDS-PAGE obtained under reducing conditions (100 mM DTT, 50°C, 15 minutes). Referring to Fig. 3, (1) and (2) represent the results for the marker and rhbFGF mutein CS23, respectively.

Fig. 4 shows the result of high performance liquid chromatography using a column (7.5 mm ID X 7.5 cm) having heparin-5PW (Tosoh, Japan) as a carrier under the following conditions:

Eluents: A solution [50 mM phosphate buffer (pH 7)]
B solution [50 mM phosphate buffer (pH 7) containing 2 M NaCl]

Flow rate: 0.8 ml/min

Detection wave length: 280 nm

Fig. 5 shows the result of high performance liquid chromatography using a column (4.6 mm ID X 150 cm) having ODP-50 (Asahi Chemical Industry, Japan) as a carrier under the following conditions:

Eluents: A solution (0.1% trifluoroacetic acid)
B solution (80% acetonitrile containing 0.1% trifluoroacetic acid)

Flow rate: 1.0 ml/min

Detection wave length: 280 nm

Fig. 6 shows the result of high performance liquid chromatography using a column (7.5 mm ID X 7.5 cm) having G2000SW (Tosoh, Japan) as a carrier under the following conditions:

Developing solution: 0.1 M phosphate buffer (pH 7)
+ 0.1 M sodium sulfate

Flow rate: 0.5 ml/min

Detection wave length: 280 nm

The biological activity was assayed by the method in which the growth promoting activity of fetal calf cardiac endothelial cells was given as an indication of the biological activity. As a result, an activity equivalent to that of the sample was shown.

Example 8

Preparation of rhbFGF Mutein CS23 Production Recombinant Having a Tetracycline Resistant Marker

Plasmid pTB762 containing a gene coding for rhbFGF mutein CS23 in which the cysteine residues at positions 69 and 87 of four cysteine residues existing in hbFGF were replaced by serine residues [Senoo et al., Biochemical and Biophysical Research Communication 151, 701- 708 (1988), European Patent Publication No. 281,822] was completely digested with AvaI and PstI to obtain a DNA fragment of about 0.45 kbase pairs containing most of rhbFGF mutein CS23. A synthetic DNA (GATCTGC / ACGTCTAGACG) was ligated to the resulting fragment with T4DNA ligase, and then the resultant was digested with AvaI and PstI to obtain a fragment A in which a PstI cleavage site was modified to a BglII cleavage site. Thereafter, plasmid pTB955 into which a gene coding for rhbFGF mutein C128 being deleted C-terminus of hbFGF [Seno et al., European Journal of

Biochemistry, 188, 239-245 (1990)] was completely digested with SalI and BamHI to obtain a fragment B of about 4.1 kbase pairs. pTB955 was completely digested with AvaI and SalI to obtain a fragment C of about 390 base pairs.

The three fragments A, B and C were ligated with T4DNA ligase each other to obtain an expression plasmid pHP901 containing an ampicillin resistant marker (Fig. 7).

pHP901 was completely digested with EcoRI and BglII to obtain a fragment of about 1.1 kbases containing a gene coding for rhbFGF mutein CS23, T7 promoter and T7 terminator. The fragment was ligated to pUC18 completely digested with EcoRI and BamHI by T4DNA ligase to obtain plasmid pME901 (Fig. 8).

pME901 was completely digested with EcoRV and HindIII to obtain a fragment of about 0.77 kbases containing a gene coding for rhbFGF mutein CS23, T7 promoter and T7 terminator. The fragment was incubated with T4DNA polymerase to change the termini thereof to flush ends. The fragment was ligated to pBR322 digested with ScaI by T4DNA ligase to obtain an expression plasmid pCM901 containing a tetracycline marker (Fig. 9).

E. coli μM294 was lysogenized with phage DE3 in which an RNA polymerase gene of the T7 phage was inserted [F. W. Studier et al., Journal of Molecular Biology 189, 113-130 (1986)] to prepare E. coli strain MM294(DE3). This E. coli strain was transformed by using the expression plasmid pCM901, thereby obtaining recombinant

E. coli MM294(DE3)/pCM901 (IFO 15104, FERM BP-3168) carrying the gene coding for rhbFGF mutein CS23.

Example 9

1 liter of LB medium containing 5 mg/l of tetracycline hydrochloride was inoculated with 1 ml of the master cells of recombinant E. coli MM294(DE3)/pCM901 (IFO 15104, FERM BP-3168), and the cultivation with shaking was carried out at 30°C for 8 hours. The total amount thereof was transferred to 20 liter of LB medium (containing 5 mg/l of tetracycline hydrochloride) placed in a 50 liter culture tank, and the cultivation was carried out at 30°C, under an inner pressure of 1.0 kg/cm^{2}G, with aeration at a rate of 10 l/min and with stirring at 300 rpm up to a Klett unit of 700. Then, the total amount of the resulting culture solution was transferred to 360 liter of a fermentation medium (M-9 medium containing 15 g/l of glucose, 15 g/l of casamino acids, 16.8 g/l of $Na_2HPO_4 \cdot 12H_2O$, 3 g/l of KH_2PO_4, 1 g/l of NH_4Cl, 0.5 g/l of sodium chloride, 0.5 g/l of $MgSO_4 \cdot 7H_2O$, 5 mg/l of thiamin hydrochloride, 2.5 mg/l of $FeSO_4 \cdot 7H_2O$ and 0.2 g/l of antifoaming agent), and the cultivation was initiated at 28°C, under an inner pressure of 1.0 kg/cm^{2}G, with aeration at a rate of 240 l/min and with stirring at 250 rpm. When the culture was grown to a Klett unit of about 450, 10 mg/l (42 μM) of IPTG was added thereto. Since the remaining sugar was about 1%, a solution containing a mixture of glucose (60 g/l) and casein hydrolysate (15 g/l) was fed and the cultivation was

continued for 40 hours. As a result, 1.6 g/l of rhbFGF mutein CS23 was accumulated in a soluble state. The cultivated product thus obtained was subjected to a Sharpless centrifuge to collect wet cells, and the cells were frozen at -80°C to store them.

1 kg of the obtained cells were treated by the manner similar to Example 7 to obtain a purified rhbFGF mutein CS23 original solution (yield 25.0 g). Quality of the solution was equal to that of the sample obtained in Example 7 in both of a biological activity and physical and chemical assay.

Incorporated by reference

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CLAIMS

WHAT IS CLAIMED IS:

1. A vector containing a nucleotide sequence coding for a mutein in which at least one constituent amino acid of a mature human basic fibroblast growth factor (hbFGF) is replaced by another amino acid, and a T7 promoter upstream therefrom.

2. The vector as claimed in claim 1, in which the at least one constituent amino acid is at least one cysteine residue and the another amino acid is a serine residue.

3. The vector as claimed in claim 1, in which the mutein is a mutein in which cysteine residues at the 69- and 87-positions of the mature hbFGF constituent amino acids are replaced by serine residues.

4. A transformant transformed by the vector as claimed in claim 1.

5. The transformant as claimed in claim 4, in which a host is Escherichia coli having a T7 RNA polymerase gene downstream from a lac promoter.

6. The transformant as claimed in claim 5, which has a characteristics of E. coli MM294(DE3)/pTB960.

7. The transformant as claimed in claim 5, which has a characteristics of E. coli MM294(DE3)/pCM901.

8. A method for producing a mutein in which at least one constituent amino acid of a mature hbFGF is replaced by another amino acid, which comprises cultivating the transformant as claimed in claim 4 in a culture m dium.

9. The method as claimed in claim 8, in which about 3 to 500 μM of isopropylthiogalactopyranoside (IPTG) is added to the culture medium on a logarithmic growth phase of the transformant as claimed in claim 5, followed by cultivation.

10. The method as claimed in claim 9, in which 3 to 300 μM of IPTG is employed.

11. The method as claimed in claim 10, in which 6 to 200 μM of IPTG is employed.

12. The method as claimed in claim 11, in which 6 to 80 μM of IPTG is employed.

13. A method as claimed in claim 8, in which a resultant mutein-containing solution is purified by chromatography using a crosslinked polysaccharide sulfate, a synthetic polymer having a sulfonic acid group as an exchange group and/or a synthetic polymer for gel filtration as a carrier.

International Application No: PCT/JP 90 / 01646

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 24, line 15 of the description [1]

A. IDENTIFICATION OF DEPOSIT[2]

Further deposits are identified on an additional sheet ☐[3]

Name of depositary Institution[4]
IFO: Institute for Fermentation, Osaka
FRI: Fermentation Research Institute, Agency of Industrial Science and Technology Ministry of International Trade and Industry

Address of depositary Institution (including postal code and country)[4]
IFO: 17-85, Juso-honmachi 2-chome, Yodogawa-ku, Osaka-shi, Osaka 532 Japan
FRI: 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki 305 Japan

Date of deposit[5]	Accession Number[6]
IFO: 26. 10. 90 FRI: 17. 11. 90	IFO-15104 FERM BP-3168

B. ADDITIONAL INDICATIONS[7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Ruel 28(4) EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE[3] (If the indications are not for all designated States)

States members of the European Patent Convention which have been designated for the purpose of a European Patent.

D. SEPARATE FURNISHING OF INDICATIONS[8] (leave blank if not applicable)

The indication listed below will be submitted to the International Bureau later[9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer)

☑ The date of receipt (from the applicant) by the International Bureau[10]

was 26 March, 1991

T. Shimomichi
(Authorized Officer)

International Application No: PCT/JP 90 / 01646

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 24, line 13 of the description [1]

A. IDENTIFICATION OF DEPOSIT[2]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution[4]
IFO: Institute for Fermentation, Osaka
FRI: Fermentation Research Institute, Agency of Industrial Science and Technology Ministry of International Trade and Industry

Address of depositary institution (including postal code and country)[4]
IFO: 17-85, Juso-honmachi 2-chome, Yodogawa-ku, Osaka-shi, Osaka 532 Japan
FRI: 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki 305 Japan

Date of deposit[5]	Accession Number[6]
IFO: 12. 12. 89 FRI: 16. 12. 89	IFO- 14979 FERM BP- 2690

B. ADDITIONAL INDICATIONS[7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Ruel 28(4) EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE[3] (if the indications are not for all designated States)

States members of the European Patent Convention which have been designated for the purpose of a European Patent.

D. SEPARATE FURNISHING OF INDICATIONS[8] (leave blank if not applicable)

The indication listed below will be submitted to the International Bureau later[9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer)

☑ The date of receipt (from the applicant) by the International Bureau[10]

was 26 March, 1991

T. Shimomichi
(Authorized Officer)

International Application No: PCT/JP 90 / 01646

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 24, line 11 of the description [1]

A. IDENTIFICATION OF DEPOSIT[2]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution[4]

IFO: Institute for Fermentation, Osaka

FRI: Fermentation Research Institute, Agency of Industrial Science and Technology Ministry of International Trade and Industry

Address of depositary institution (including postal code and country)[4]

IFO: 17-85, Juso-honmachi 2-chome, Yodogawa-ku, Osaka-shi, Osaka 532 Japan

FRI: 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki 305 Japan

Date of deposit [5]	Accession Number [6]
IFO: 27. 05. 87	IFO- 14613
FRI: 11. 06. 87	FERM BP- 1645

B. ADDITIONAL INDICATIONS[7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Ruel 28(4) EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [3] (if the indications are not for all designated States)

States members of the European Patent Convention which have been designated for the purpose of a European Patent.

D. SEPARATE FURNISHING OF INDICATIONS[8] (leave blank if not applicable).

The indication listed below will be submitted to the International Bureau later[9] (Specify the general nature of the indications .g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer)

☑ The date of receipt (from the applicant) by the International Bureau[10]

was 26 March, 1991

T. Shimomichi

(Authorized Officer)

International Application No: PCT/JP 90 / 01646

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 24, line 17 of the description [1]

A. IDENTIFICATION OF DEPOSIT[2]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution[4]
IFO: Institute for Fermentation, Osaka
FRI: Fermentation Research Institute, Agency of Industrial Science and Technology Ministry of International Trade and Industry

Address of depositary institution (including postal code and country)[4]
IFO: 17-85, Juso-honmachi 2-chome, Yodogawa-ku, Osaka-shi, Osaka 532 Japan
FRI: 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki 305 Japan

Date of deposit [5]	Accession Number [6]
IFO: 02. 02. 89 FRI: 13. 02. 89	IFO- 14827 FERM BP- 2283

B. ADDITIONAL INDICATIONS[7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Ruel 28(4) EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [3] (if the indications are not for all designated States)

States members of the European Patent Convention which have been designated for the purpose of a European Patent.

D. SEPARATE FURNISHING OF INDICATIONS[8] (leave blank if not applicable)

The indication listed below will be submitted to the International Bureau later[9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer)

☑ The date of receipt (from the applicant) by the International Bureau[10]

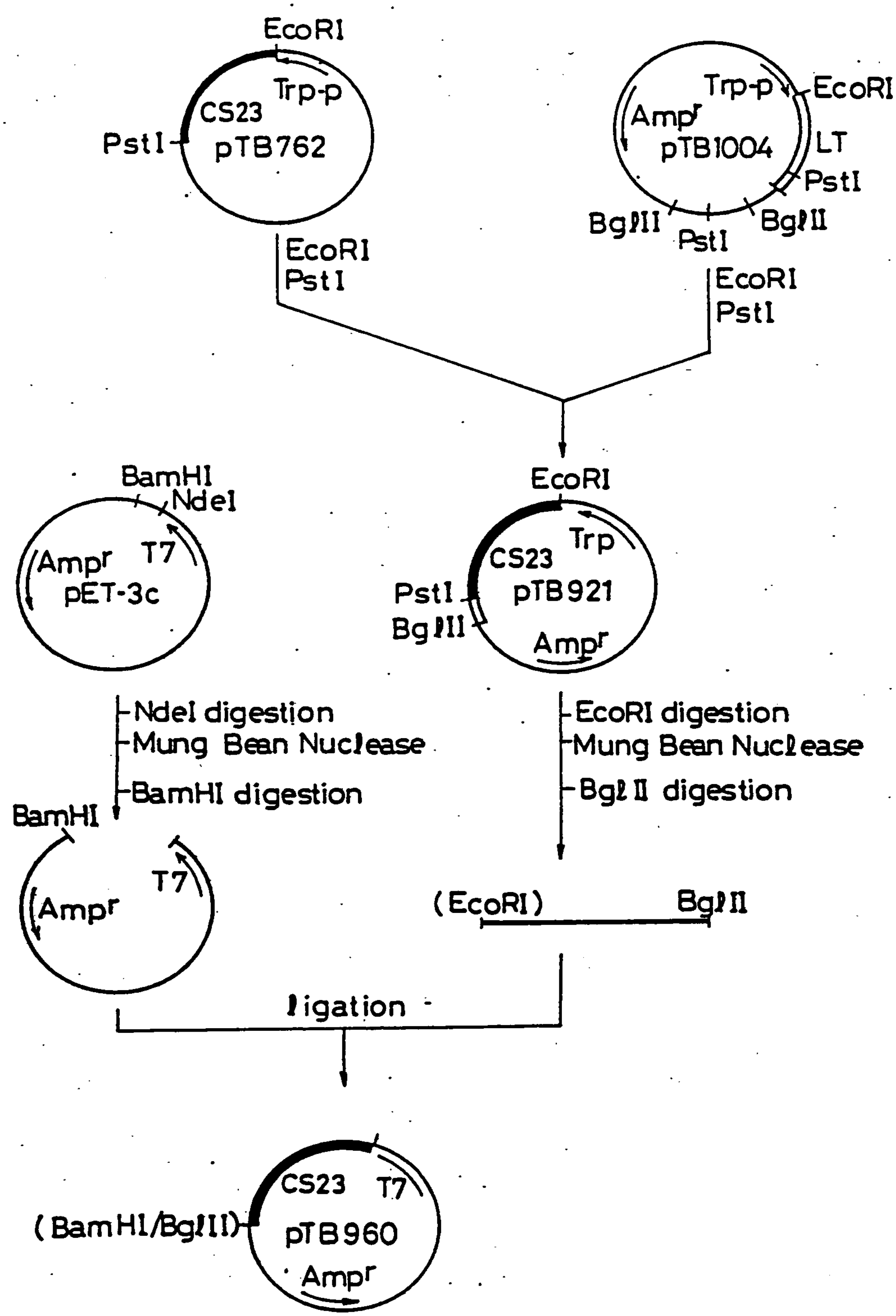
was 26 March, 1991

T. Shimomichi
(Authorized Officer)

Fig. 1

```
MetProAlaLeuProGluAspGlyGlySerGlyAlaPheProProGlyHisPheLysAsp     20
ATGCCAGCATTGCCCGAGGATGGCGGCAGCGGCGCCTTCCCGCCCGGCCACTTCAAGGAC     60

ProLysArgLeuTyrCysLysAsnGlyGlyPhePheLeuArgIleHisProAspGlyArg     40
CCCAAGCGGCTGTACTGCAAAAACGGGGGCTTCTTCCTGCGCATCCACCCCGACGGCCGA    120

ValAspGlyValArgGluLysSerAspProHisIleLysLeuGlnLeuGlnAlaGluGlu     60
GTTGACGGGGTCCGGGAGAAGAGCGACCCTCACATCAAGCTACAACTTCAAGCAGAAGAG    180

ArgGlyValValSerIleLysGlyValSerAlaAsnArgTyrLeuAlaMetLysGluAsp     80
AGAGGAGTTGTGTCTATCAAAGGAGTGAGCGCTAATCGTTACCTGGCTATGAAGGAAGAT    240

GlyArgLeuLeuAlaSerLysSerValThrAspGluCysPhePhePheGluArgLeuGlu    100
GGAAGATTACTAGCTTCTAAGTCTGTTACGGATGAGTGTTTCTTTTTTGAACGATTGGAA    300

SerAsnAsnTyrAsnThrTyrArgSerArgLysTyrThrSerTrpTyrValAlaLeuLys    120
TCTAATAACTACAATACTTACCGGTCAAGGAAATACACCAGTTGGTATGTGGCACTGAAA    360

ArgThrGlyGlnTyrLysLeuGlySerLysThrGlyProGlyGlnLysAlaIleLeuPhe    140
CGAACTGGGCAGTATAAACTTGGATCCAAAACAGGACCTGGGCAGAAAGCTATACTTTTT    420

LeuProMetSerAlaLysSertrm                                        147
CTTCCAATGTCTGCTAAGAGCTGA                                        444
```

FIG.2



EcoRI
Trp-p
CS23
PstI
pTB762
Trp-p
EcoRI
Ampr
pTB1004
LT
PstI
BglII
PstI
BglII
EcoRI
PstI
EcoRI
PstI
BamHI
NdeI
Ampr
T7
pET-3c
EcoRI
Trp
CS23
PstI
pTB921
BglII
Ampr
NdeI digestion
Mung Bean Nuclease
BamHI digestion
EcoRI digestion
Mung Bean Nuclease
BglII digestion
BamHI
T7
Ampr
(EcoRI)
BglII
ligation
CS23
T7
(BamHI/BglII)
pTB960
Ampr

FIG.3

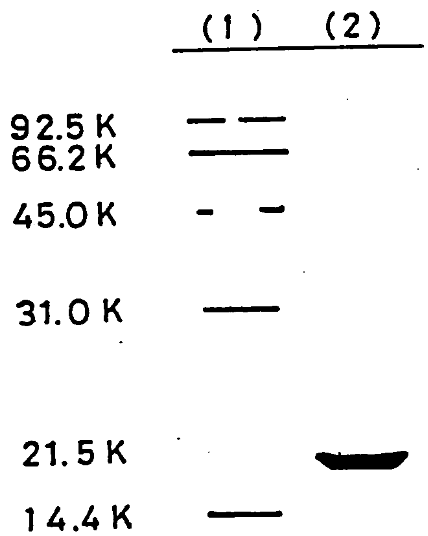


FIG.4

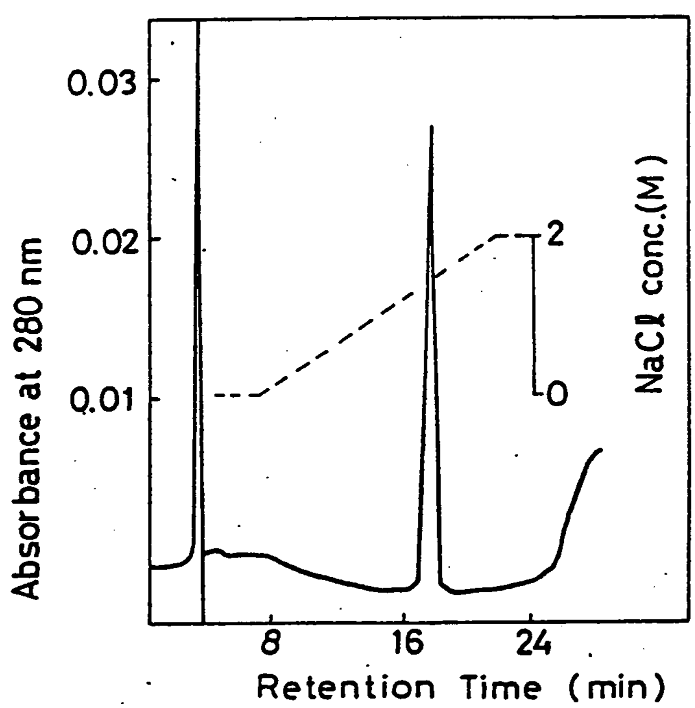
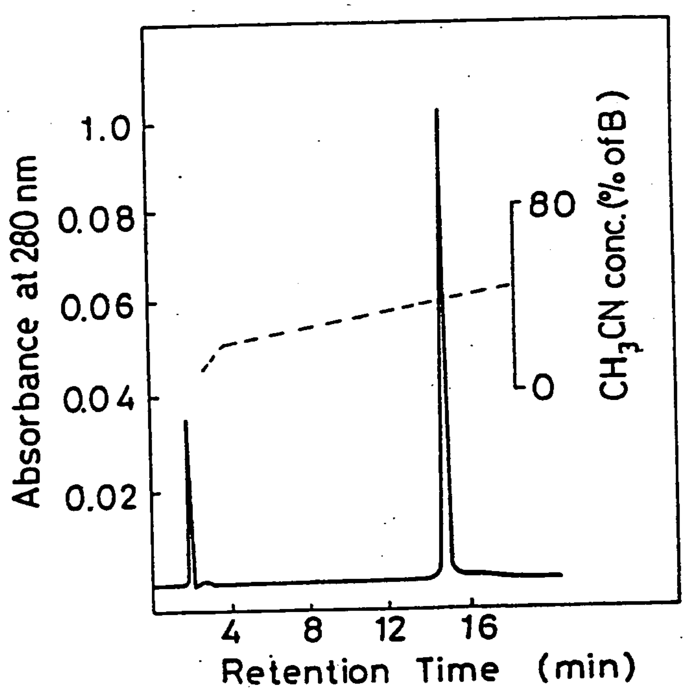

0.03
0.02
0.01
Absorbance at 280 nm
2
0
NaCl conc.(M)
8
16
24
Retention Time (min)

FIG.5



Absorbance at 280 nm
1.0
0.08
0.06
0.04
0.02
80
0
CH3CN conc.(% of B)
4
8
12
16
Retention Time (min)

FIG.6

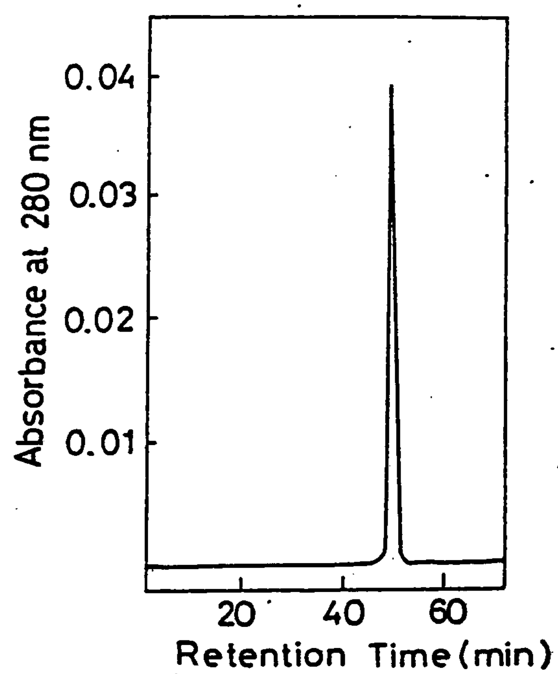

0.04
0.03
0.02
0.01
Absorbance at 280 nm
20
40
60
Retention Time (min)

FIG.7

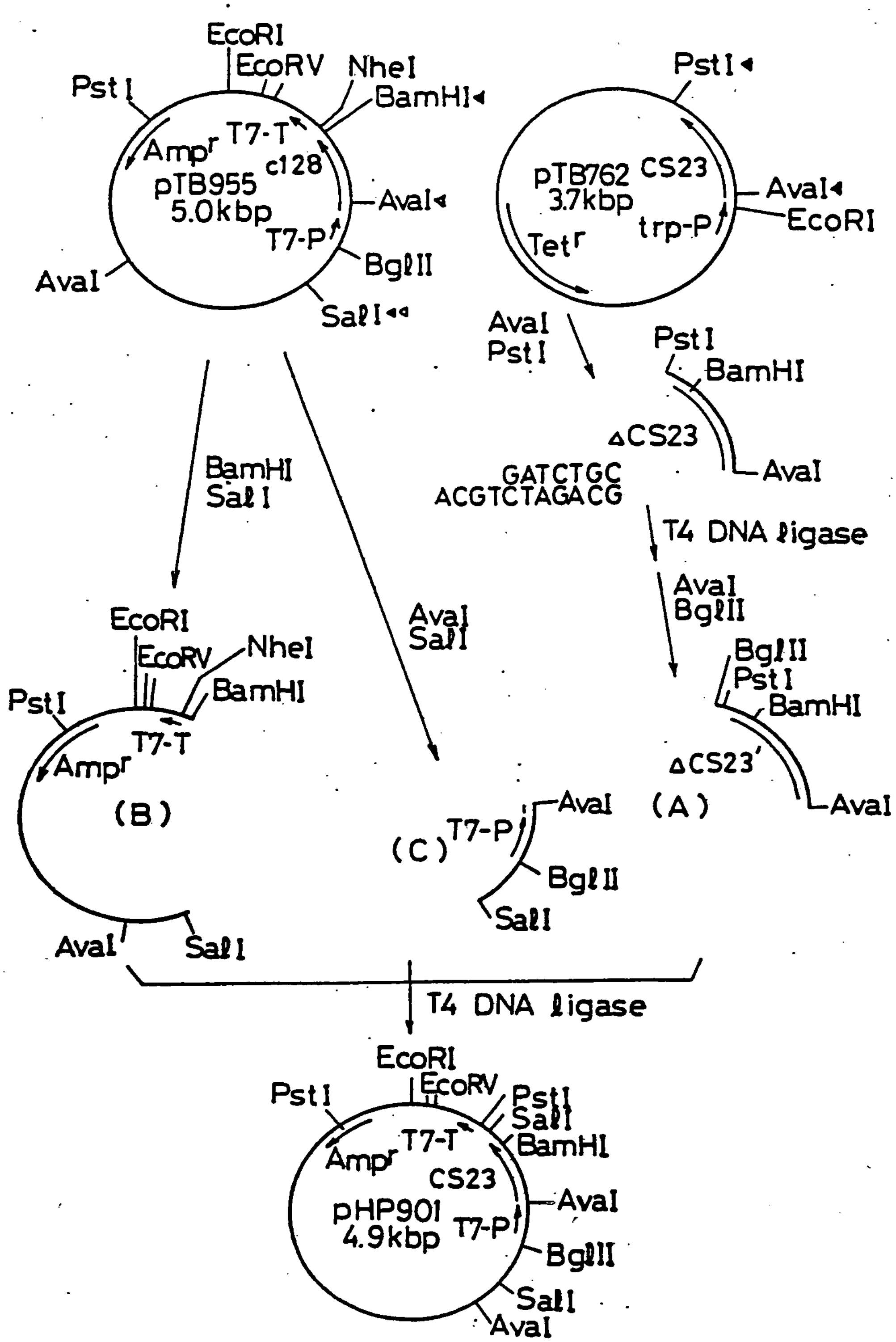

EcoRI
EcoRV
NheI
BamHI
PstI
Amp^r
T7-T
c128
pTB955
5.0kbp
AvaI
T7-P
BglII
AvaI
SalI
PstI
pTB762
3.7kbp
CS23
AvaI
EcoRI
trp-P
Tet^r
AvaI
PstI
PstI
BamHI
ΔCS23
GATCTGC
ACGTCTAGACG
AvaI
T4 DNA ligase
AvaI
BglII
BamHI
SalI
AvaI
SalI
BglII
PstI
BamHI
ΔCS23'
AvaI
(A)
EcoRI
EcoRV
NheI
BamHI
PstI
T7-T
Amp^r
(B)
AvaI
SalI
(C)
T7-P
AvaI
BglII
SalI
T4 DNA ligase
EcoRI
EcoRV
PstI
SalI
BamHI
PstI
T7-T
Amp^r
CS23
pHP901
4.9kbp
T7-P
AvaI
BglII
SalI
AvaI

FIG.8

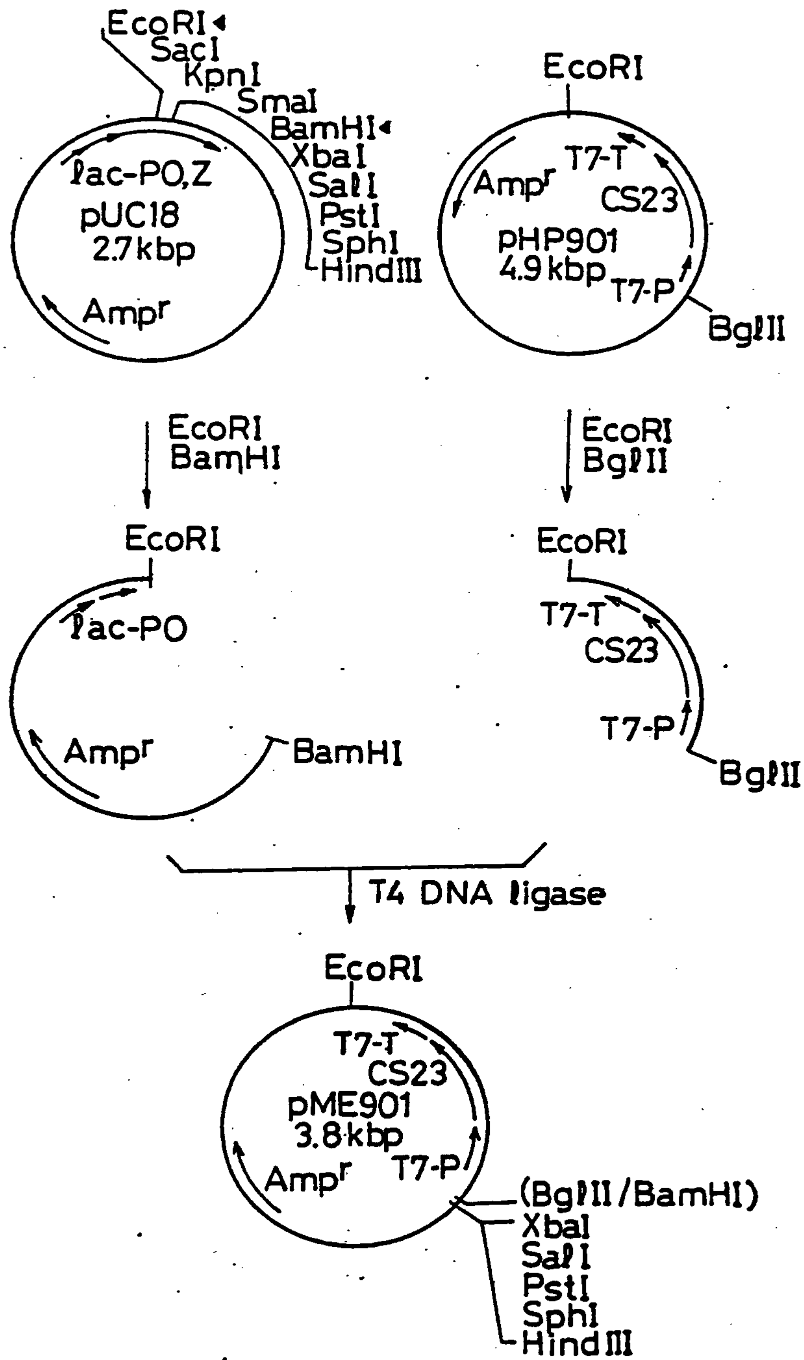
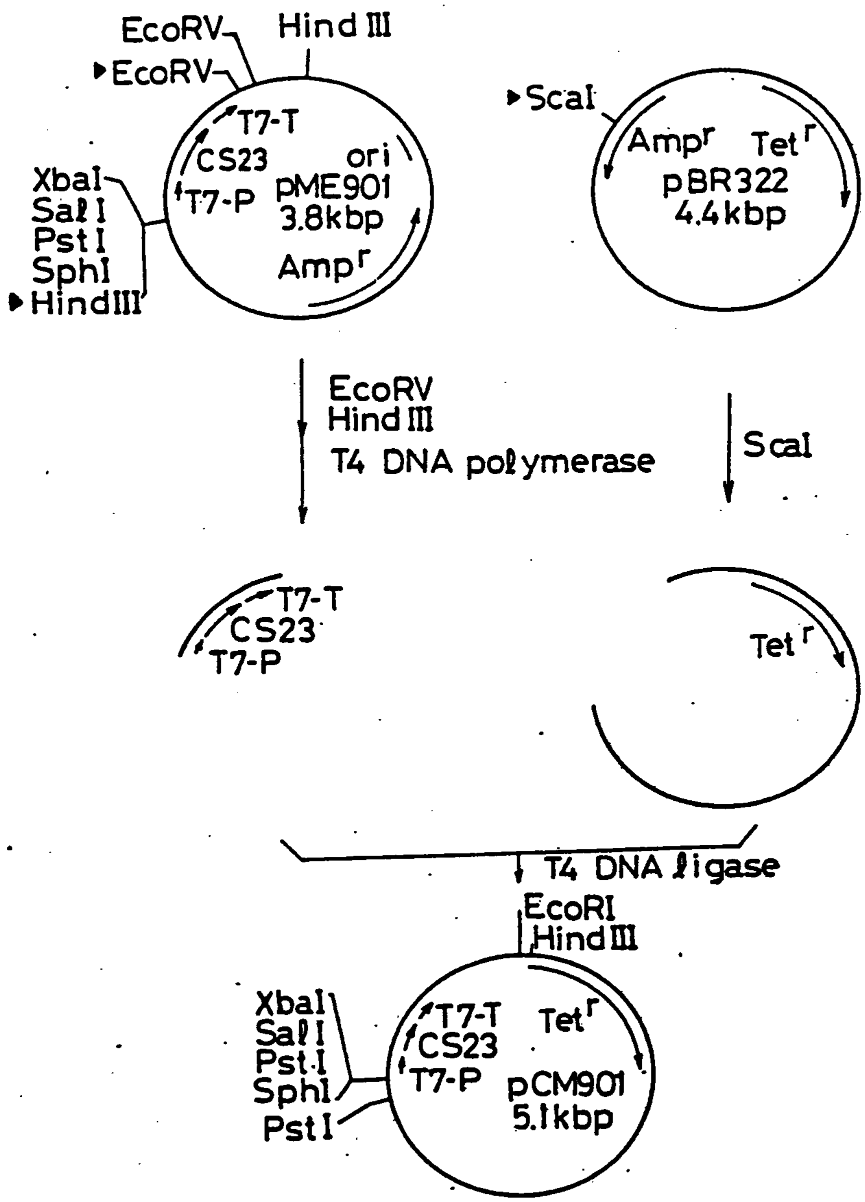

EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
SphI
HindIII
lac-PO,Z
pUC18
2.7kbp
Ampr
EcoRI
T7-T
Ampr
CS23
pHP901
4.9kbp
T7-P
BglII
EcoRI
BamHI
EcoRI
BglII
EcoRI
lac-PO
Ampr
BamHI
EcoRI
T7-T
CS23
T7-P
BglII
T4 DNA ligase
EcoRI
T7-T
CS23
pME901
3.8kbp
Ampr
T7-P
(BglII/BamHI)
XbaI
SalI
PstI
SphI
HindIII

FIG.9


EcoRV
EcoRV
Hind III
T7-T
CS23
T7-P
ori
pME901
3.8kbp
Ampʳ
XbaI
SalI
PstI
SphI
HindIII
ScaI
Ampʳ
Tetʳ
pBR322
4.4kbp
EcoRV
Hind III
T4 DNA polymerase
ScaI
T7-T
CS23
T7-P
Tetʳ
T4 DNA ligase
EcoRI
Hind III
XbaI
SalI
PstI
SphI
PstI
T7-T
CS23
T7-P
Tetʳ
pCM901
5.1kbp

INTERNATIONAL SEARCH REPORT

International Application No PCT/JP 90/01646

I. CLASSIFICATI N OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC^5: C 12 N 15/16, C 12 N 1/21, C 12 N 15/70, C 12 N 15/67

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC^5	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[*]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	EP, A, 0326907 (TAKEDA CHEMICAL LTD) 9 August 1989 see page 21 - page 23 (examples 6,7, claims); page 8, lines 30-46 --	1-13
Y	EP, A, 0320148 (AMGEN INC.) 14 June 1989 see claims --	1-13
Y	EP, A, 0300425 (BOEHRINGER MANNHEIM GmbH) 25 January 1989 see claims; page 3, lines 11-57 --	1-13
Y	EP, A, 0178863 (SCHERING CORP.) 23 April 1986 see claims ------------	1-13

* Special categories of cited documents:[10]
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
4th March 1991	28.03.91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	p.p. MISS D. S. KOWALCZYK

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. JP 9001646 SA 42647

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 25/03/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0326907	09-08-89	JP-A-	2000193	05-01-90
		AU-A-	1208588	25-08-88
		EP-A-	0281822	14-09-88
		EP-A-	0288687	02-11-88
		JP-A-	2209894	21-08-90
EP-A- 0320148	14-06-89	AU-A-	2818189	14-06-89
		WO-A-	8904832	01-06-89
EP-A- 0300425	25-01-89	DE-A-	3723992	02-02-89
		JP-A-	1043195	15-02-89
EP-A- 0178863	23-04-86	JP-A-	61100197	19-05-86

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82